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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Venkat Gopalan et al.	Art Unit:	1652
Serial No.:	09/516,061	Examiner:	Charles L. Patterson Jr.
Filed:	March 1, 2000	Customer No.:	21559

Title: NOVEL BACTERIAL RNASE P PROTEINS AND THEIR USE IN IDENTIFYING ANTIBACTERIAL COMPOUNDS

Commissioner For Patents
P.O. Box 1450
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DECLARATION OF DR. VENKAT GOPALAN UNDER 37 C.F.R. § 1.131

I, Venkat Gopalan, declare that:

1. I am an inventor of the invention described and claimed in the above-identified patent application.

2. In response to the Examiner's concern that some of the bacterial RNase P polypeptides do not have accession numbers despite the ability of bacteria to have more than one RNase P polypeptide, I note there is only one RNase P polypeptide in each bacterial species. The different accession numbers for some RNase P polypeptide sequences is due to multiple deposits of the same sequence.

3. A skilled artisan can easily measure the rate of hydrolysis f an RNase P substrate by an RNase P holoezyme of interest and determine whether that rate is at least 20% of the rate of hydrolysis of the same RNase P substrate by the same concentration of an *E. coli* or *B. subtilis* RNase P holoenzyme under the same conditions, using standard assays such as those disclosed on pages 19-23 of the specification.

For example, the specification teaches:

> [b]y "a polypeptide containing RNase P activity" is meant a polypeptide sequence that, when combined with an RNA subunit to form an RNase P holoenzyme, has 20%, 50%, 75%, or even 100% or more, of the enzymatic activity of an *E. coli* or *B. subtilis* RNase P holoenzyme. Preferably, the RNA subunit is from the same species when activity is tested. The enzymatic activity can be assessed, for example, by measuring *hydrolysis of an RNase P substrate*. Standard methods for conducting such hydrolysis assays are described herein and in the literature (see, e.g., Altman and Kirsebom, Ribonuclease P, *The RNA World*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999; Pascual and Vioque, Proc. Natl. Acad. Sci. 96: 6672, 1999; Geurrier-Takada et al., Cell 35: 849, 1983; Tallsjö and Kirsebom, Nucleic Acids Research 21: 51, 1993; Peck-Miller and Altman, J. Mol. Biol. 221: 1, 1991; Gopalan et al., J. Mol. Biol. 267: 818, 1997; and WO 99/11653).
>
> By "RNase P substrate" is meant a substrate in which hydrolysis by an RNase P holoenzyme requires the presence of the RNase P protein subunit. (page 4, line 25 through page 5, line 10, emphasis added)

The specification also teaches the following exemplary assays and reaction buffers:

> [s]amples of the RNase P holoenzyme and the RNase P substrate are mixed, incubated, and measured for spectrophotometric polarization. When the substrate is cleaved by the RNase P holoenzyme, the 10-nucleotide 5'- leader sequence is released, which leads to a substantial change in the fluorescence polarization in the sample. (Campbell, I.D. & Dwed., R.A. pp. 91-125 The Benjamin/Cummings Publishing Company, Menlo Park, CA (1984); Lakowicz, J.R., Plenum Press, NY (1983)).
>
> The preferred reaction buffer contains 50 mM Tris-HCl (pH 7.5), 100 mM ammonium chloride and 10 mM magnesium chloride. Concentrations of 10-100 mM, 25-500 mM and 1-100 mM of the above, respectively, can be substituted, as can other buffering agents such as MOPS or HEPES, or other monovalent cations, such as sodium or potassium. When the assay is run in either 96 or 384-well polystyrene or p lypropylene assay plates, there is a very significant decrease in the fluorescence intensity and polarization of the annealed substrate over time in

the absence of enzyme. Various conditions have been tested to prevent the loss of signal with time. The preferred conditions include addition of 10-40 µg/ml carbonic anhydrase and 10-100 µg/ml polyC to the buffer. Other materials, such as, 0.5-5% glycerol, 10-100 µg/ml hen egg lysozyme, 10-50 µg/mL tRNA, 1-10 mM DTT, or 2-10 mM DTT can also be added to the buffer to prevent some loss of signal. (page 22, lines 3-21)

Exhibit 1 is a picture of a gel showing the results of an exemplary assay in which the activity of an RNase P polypeptide of interest (*Neisseria gonorrhoaea*) was compared to that of *E. coli* RNase P to determine whether the activity of *N. gonorrhoaea* RNase P is at least 20% of the activity of *E. coli* RNase P. The reaction mixture included 1 nM RNase P RNA subunit, 1-5 nM RNase P protein subunit, 40 nM pre-tRNA substrate, 50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, and 10 mM $MgCl_2$ at room temperature (22 °C). The reaction was allowed to proceed for 5-60 minutes.

The RNase P substrate was the 85-nucleotide pre-$tRNA^{Gln}$ from Synechocystis. The substrate was labeled with ^{32}P at one position at the 5'end. Therefore, only the full-length precursor of 85 nucleotides and the 5' processed leader sequence of 10 nucleotides are detectable on this autoradiogram. The 75-nucleotide mature product is not labeled, and therefore not detectable. Lanes labeled "E" contain samples from reactions incubated with the *E. coli* holoenzyme, and those labeled "N" contain samples from reactions incubated with the *N. gonorrhoeae* holoenzyme. Each pair of lanes represents a particular incubation time in minutes (denoted above the lanes).

4. With respect to isolation methodologies for RNase polypeptides, clear instructions for isolating other claimed RNase P polypeptides are provided in the specification on pages 10-17.

Any "experimentation" involved in isolating and characterizing additional RNase P polypeptides falling within the present claims is straightforward, and is rendered so by the discovery of the RNase P consensus sequence and 19 RNase P polypeptide s quences. At the time of filing, a skilled artisan, using no more than routine experimentation and the teachings of the present specification, could easily identify other

polypeptides having an RNase P consensus sequence and assay them for RNase P activity using standard techniques. For example, the specification teaches:

> [b]y "an RNase P consensus sequence" is meant a sequence which, when aligned to the *E. coli* RNase P sequence using the ClustalW program and performing a comparison of the specified amino acid sequences, shows conservation of at least nine of the following specified 20 amino acid residues in the *E. coli* RNase P protein subunit: R11, L12, F18, R46, G48, V51, K53, K54, A59, V60, R62, N63, K66, R67, R70, L80, D84, V86, L101, and L105. Preferably, the consensus sequence conserves at least 13 of the 20 residues. It is also preferred that the aligned consensus sequence contain at least seven of the following subset of nine amino acid residues in the *E. coli* RNase P protein: F18, R46, K53, A59, R62, N63, K66, R67, R70, more preferably, at least eight of the amino acids, and, most preferably, all nine amino acids of the above subset. For the purpose of determining identity in the present invention, identity of amino acids or other than those for which the amino acid is specified in the consensus sequence are ignored in the comparison when calculating identity of nucleic acids encoding an RNase P consensus sequence degenerate codons encoding the designated amino acid are treated as identical. (page 7, lines 1-16)

Based on these teaching, one skilled in the art can readily determine whether a polypeptide has an RNase P consensus sequence. If desired, the RNase P activity of the polypeptide can be confirmed using any of the assays taught in the specification (see, for example, pages 5 and 19-23).

Exhibit 2 illustrates the ability of recombinant *N. gonorrhoaea* RNase P to cleave an 85-nucleotide pre-$tRNA^{Gln}$ substrate that was uniformly radiolabeled with ^{32}P at the G positions. The reaction mixture included 1 nM RNase P RNA subunit, 1-5 nM RNase P protein subunit, 40 nM pre-tRNA substrate, 50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, and 10 mM $MgCl_2$ at room temperature (22 °C). The reaction was allowed to proceed for 5-60 minutes.

Exhibit 3 illustrates the ability of recombinant *Porphyromonas gingivalis* RNase P to cleave pre-$tRNA^{Gln}$. The reaction conditions were similar to those for Exhibit 2, except that the enzyme concentration was10 nM, and the reaction time was 10 minutes. These results confirm that polypeptides with the RNase P consensus sequenc have RNase P activity.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 08/04/2003

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